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| <p>(54) Title: PREPARATION OF PHOTOPROTEIN CONJUGATES AND METHODS OF USE THEREOF</p> <p>(57) Abstract</p> <p>The present invention encompasses a method of synthesis of conjugates of photoproteins that retain all or a substantial portion of the luminescent activity of underivatized photoprotein. According to the present invention photoproteins may be conjugated with a variety of binding reagent including streptavidin/avidin, glycoproteins, lectins, hormones, antigens, drugs, antibodies and antigen binding fragments thereof, or any other selectively bindable reagent by chemical cross-linking means. The present invention also encompasses conjugates produced by this method, and methods of use of such conjugates.</p> | | | |

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**PREPARATION OF PHOTOPROTEIN
CONJUGATES AND METHODS OF USE THEREOF**

Technical Field

15 The invention relates to labeled specific-binding assay reagents, and more particularly to specific-binding assay reagents comprising luminescent proteins coupled to binding molecules.

Background of the Invention

20 Bioluminescence refers to the emission of light by biological molecules. Bioluminescent proteins can be true enzymes such as luciferases, which catalyze the oxidation of luciferin, emitting light and releasing oxyluciferin, or photoproteins, which catalyze the oxidation of luciferin to emit light but do not release the oxidized substrate. Examples of 25 bioluminescent proteins include those isolated from the ctenophores *Mnemiopsis* (*mnemiopsin*) and *Beroe ovata* (*berovin*), those isolated from the coelenterates *Aequoria* (*aequorin*), *Obelia* (*obelin*), *Pelagia*, and luciferases such as *Renilla* (*Renilla luciferase*) and those isolated from the molusca *Pholas* (*pholasin*). Bioluminescent proteins also can be isolated 30 from ostracods such as *Cypridina*.

35 A bioluminescent protein particularly useful as a label in diagnostic assays is the photoprotein aequorin. Aequorin catalyzes the oxidation of coelenterate luciferin to oxyluciferin, resulting in the concomitant production of blue light ($\lambda_{max} = 469$ nm). Aequorin consists of apoaequorin, a single polypeptide chain of M_r 22,000, containing one mole each of tightly bound coelenterate luciferin and oxygen. Light emission from this complex

is initiated upon the binding of calcium ions. Aequorin catalyzes a single turnover event or "flash reaction" which persists for approximately 10 seconds.

5 Due to the relatively high quantum yield of the reaction, aequorin can be detected at the attomol level (10^{-18} mol) using commercially available luminometers. Because of its calcium requirement and ability to be detected at very low levels, aequorin has proven useful for monitoring levels of intracellular calcium. The potential utility of using aequorin as a nonradioisotopic reporter molecule has only recently been realized with the 10 availability of the recombinant protein (reviewed by Cormier et al., Cormier, M.J. Prasher DC Longiatu, M & McCann, R.O. 1989, *Photochem. Photobiol.* 49: 509-512).

15 Until very recently, it was generally considered in the art that all bioluminescent proteins were inherently unstable and complex, thus making them unsuitable for reporter molecules, based on the properties of firefly *Photinus pyralis* luciferase (Bronstein & McGrath, 1989 Chemiluminescence Lights Up. *Nature* 338, 599-600). However, the biotinylated derivative of aequorin recently has been shown to retain greater than 80 percent of its original activity and, when used in combination with 20 streptavidin, has been demonstrated to detect nanogram to subnanogram amounts of biotinylated targets, including protein antigens and DNA immobilized onto microtiter walls or membrane supports (Stults et al., 1992, Rivera, H., McCann, R.O., O'Kane D., Cummings, R.D., Cormier M.J., Smith D.F. Use of Recombinant Biotinylated-Aequorin in Microtiter 25 and Membrane-Based Assays. Purification of Recombinant Apoaequorin From *Escherichia coli*. *Biochemistry*, 31, 1433-1442). In addition, biotinylated aequorin also has been successfully used in a capture immunoassay for *Salmonella* antigen (Smith et al., 1991, A Microplate Assay For Analysis of Solution Phase Glycosyltransferase Reactions: 30 Determination of Kinetic Constants. *Anal. Biochem.* 199, 286-292) and in solid phase assays for glycosyl transferases and glycoproteins (Mengeling et al., 1991, A Microplate Assay For Analysis of Solution Phase Glycosyltransferase Reactions: Determination of Kinetic Constants. *Anal. Biochem.* 199, 286-292), (Zatta et al., 1991, A Solid Phase Assay For 35 B1,4 Galactosyl Transferase Activity in Human Serum Using Recombinant Aequorin, *Anal. Biochem.* 194, 185-191).

5 Although the biotinylated derivative of aequorin performs well in a variety of different assay formats, it would be advantageous to synthesize by chemical crosslinking methods direct conjugates of recombinant aequorin with a variety of selectively bindable reagent including receptors, hormones, lectins, antibodies and binding fragments thereof, antigens, DNA, RNA, oligonucleotides, and glycoproteins. Such conjugates would facilitate faster detection of biological targets by reducing the number of incubation steps or by eliminating the avidin/streptavidin-biotin interaction entirely.

10 10 Direct chemical crosslinking of apoaequorin to antibody FAB fragments using a heterobifunctional reagent has been reported (Erikaku et al., 1991, Bioluminescent Immunoassay Using a Monomeric Fab'-Photoprotein Aequorin Conjugate. *Biochem. Biophys. Res. Commun.* 174, 1331-1336). The antibody conjugate was prepared using a chemical crosslinking technique based on modification of amino or thiol groups on apoaequorin with maleimido groups. Unfortunately, this crosslinking procedure results in apoaequorin-antibody conjugates that retained about only 10 percent of the activity of underivatized aequorin. (See Abst., p. 1331 Erikaku et al, 1991).

15 15 20 What is needed, therefore, is a direct chemical crosslinking method for preparing photoprotein conjugates that substantially preserves photoprotein activity.

Summary of the Invention

20 25 The present invention encompasses a method of synthesis of conjugates of photoproteins that retain a substantial portion of the luminescent activity of underivatized photoprotein. According to the present invention, photoproteins may be conjugated with a variety of binding reagent including, but not limited to, streptavidin/avidin, lectins, enzymes, glycoproteins, peptides, hormones, receptors, antigens, drugs, antibodies and antigen binding fragments thereof, RNA, DNA, oligonucleotides, or any other selectively bindable molecules or compositions by chemical crosslinking means.

25 30 35 The present invention also encompasses conjugates produced by this method, and methods of use of such conjugates in binding assays to determine the presence and amount in a sample of an analyte. The conjugation method of the present invention includes the steps of

introducing additional sulphhydryl groups into a photoprotein to be conjugated, chemically modifying a binding reagent to render it sulphhydryl-reactive, and reacting the sulphhydryl-activated photoprotein with the sulphhydryl-reactive binding reagent to form the photoprotein-binding reagent conjugates of the present invention. The method yields stable photoprotein-binding reagent conjugates that retain luminescent activity.

Accordingly, it is an object of the present invention to provide a method for producing stable photoprotein-binding reagent conjugates that retain bioluminescent activity.

It is a further object of the present invention to provide stable photoprotein-binding reagent conjugates that retain bioluminescent activity.

It is yet a further object of the present invention to provide a method for the production of stable conjugates of apoaequorin or aequorin that retain bioluminescent activity.

It is a still further object of the present invention to provide stable conjugates of apoaequorin or aequorin which retain bioluminescent activity.

It is yet a further object of the present invention to provide a method whereby binding reagents that contain one or more amino groups may be conjugated to a photoprotein modified to contain at least one additional sulphhydryl group such that the resulting conjugate retains bioluminescent activity.

It is also an object of the present invention to provide stable photoprotein conjugates where the binding reagent is an antibody.

It is also an object of the present invention to provide stable photoprotein conjugates where the binding reagent is an antigen.

It is an object of the present invention to provide stable photoprotein conjugates where the binding reagent is a lectin.

It is another object of the present invention to provide stable photoprotein conjugates where the binding reagent is a receptor.

It is also an object of the present invention to provide stable photoprotein conjugates where the binding reagent is a ligand.

It is also an object of the present invention to provide stable photoprotein conjugates where the binding reagent is an oligonucleotide.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiment and the appended claims.

Brief Description of the Figures

Figure 1 is a graphic illustration of the inactivation of aequorin by treatment with increasing amounts of sulfo-SMCC as measured by the amount of light produced.

Figure 2 is a graphic illustration of the lack of detrimental effect of 2-iminothiolane on aequorin as measured by the amount of light produced.

Figure 3 is a graphic illustration of aequorin-(mouse antibody) conjugates binding specifically to immobilized goat anti-mouse antibody as measured by the amount of light produced.

Figure 4 is a gel-filtration chromatographic profile illustrating the purification of aequorin-antibody conjugates as measured by light production and O.D.280 absorbance.

Figure 5 is a DEAE ion-exchange chromatographic profile illustrating the purification of aequorin-antibody conjugates as measured by light production and optical density at 280 nm (O.D.280).

Figure 6 is the graphic representation of the binding of an aequorin-thyroxine (T4) conjugate versus thyroxine (T4) in a competitive immunoassay.

Detailed Description of the Invention

Important terms used in the specification are defined as follows:

The term "photoprotein" as used herein means any bioluminescent protein, i.e. any protein that is capable of producing light or which is a component of a luminescent reaction (i.e. a bioluminescent protein-mediated reaction that generates light). The term "photoprotein" includes bioluminescent enzymes such as luciferases and photoproteins such as aequorin. Examples of photoproteins include, but are not limited to, apoaequorin, aequorin, apo-obelin, obelin, apo-mnemiopsin, mnemiopsin, apo-berovin, berovin, pholasin, and the bioluminescent proteins isolated from the organisms *Pelagia*, ostracods such as *Cypridina*, and *Renilla* luciferase. Apo- forms of the photoproteins indicate that the protein is bound luciferin. In contrast, the non-apo- forms of the photoproteins indicate that luciferin is bound to the protein.

The term "apoaequorin," as used herein, means any of the related amino acid sequences commonly known in the art as apoaequorin

5 sequences, including recombinant apoaequorin, as exemplified by Prasher et al. (1987), Sequence Comparisons of Complementary DNAs Encoding Aequorin Isotypes, *Biochemistry*, volume 26, number 5 1326-1332, which is hereby incorporated by reference. The term "apoaequorin" also is intended to encompass modified proteins, such as those altered by site-directed mutagenesis. Apoaequorin refers to the protein sequence alone without luciferin being tightly associated to form an aequorin complex.

10 The term "aequorin" as used herein means a complex formed of either naturally derived or recombinant apoaequorin protein to which luciferin and oxygen is bound. This complex is capable of producing light when triggered by a divalent cation such as calcium. The term "sulphydryl-activated photoprotein" as used herein means a photoprotein as defined above that is chemically modified to contain at least one sulphydryl group more than is present in the unmodified photoprotein.

15 The term "binding reagent," as used herein, means a compound capable of binding specifically to an analyte to be assayed that has amino groups available for chemical modification. The analyte to be assayed may be any selectively bindable material. Examples of binding reagents include, but are not limited to, streptavidin/avidin, lectins, enzymes, glycoproteins, 20 peptides, hormones, receptors, antigens, drugs, antibodies and antigen binding fragments thereof, RNA, DNA, oligonucleotides, or any other selectively bindable molecules or compositions.

25 The term "sulphydryl-activated" or "sulphydryl-activated photoprotein," as used herein, means a photoprotein that has been modified to contain at least one sulphydryl group in addition to the naturally occurring endogenous sulphydryl groups in the photoprotein. Sulphydryl-activated photoproteins can be reacted with sulphydryl-reactive binding reagents to form the conjugates of the present invention.

30 The term "sulphydryl-reactive" or "sulphydryl-reactive binding reagent," as used herein, means a binding reagent capable of reacting with a sulphydryl group on a sulphydryl-activated photoprotein to form a photoprotein-binding reagent conjugate. One class of sulphydryl-reactive binding reagents are those that have been modified to contain a maleimido group, and are termed "maleimido-activated." Another class of sulphydryl-reactive binding reagents are those that have been modified to contain an α -halocarbonyl group.

The term "photoprotein-binding reagent conjugate" or "conjugate," as used herein, means a conjugate of a photoprotein and a binding reagent, as defined above, that are covalently coupled through a sulphydryl-based crosslinking reaction.

5 The present invention encompasses a method of synthesis of conjugates of photoproteins with a variety of binding proteins including streptavidin/avidin, lectins, hormones, antigens, drugs, antibodies and antigen binding fragments thereof, or any other binding reagent by chemical crosslinking means. The present invention also encompasses photoprotein-
10 binding reagent conjugates produced by this method.

15 The conjugation method of the present invention includes the steps of introducing additional sulphydryl groups into a photoprotein to be conjugated, chemically modifying a binding reagent to render it sulphydryl-reactive, and reacting the sulphydryl-activated photoprotein with the sulphydryl-reactive binding reagent to form the photoprotein-binding reagent conjugates of the present invention. The method yields stable photoprotein-
20 binding reagent conjugates that retain luminescent activity.

25 While not wanting to be limited to this theory, it is postulated that the sulphydryl activation of the photoprotein serves to protect endogenous sulphydryl groups that are essential for bioluminescent activity. The subsequent crosslinking to a binding reagent via sulphydryl groups in the sulphydryl-activated photoprotein, does not, therefore, substantially adversely affect bioluminescent activity. This is a substantial improvement over the prior art conjugates of non-sulphydryl-activated photoproteins.

Photoproteins used within the present invention include, but are not limited to, apoaequorin, aequorin, obelin, mnemiopsis, berovin, pholasin, luciferases, and the photoproteins isolated from *Pelagia*, *Cypridina*, and ostracods. A preferred photoprotein is aequorin or apoaequorin.

30 Photoproteins are sulphydryl activated by chemical modification with appropriate reagents to add at least one more sulphydryl group than is present on the unmodified photoprotein. Preferably multiple sulphydryl groups are added to the photoprotein. Examples of such sulphydryl activating reagents include, but are not limited to, 2-iminothiolane (Traut's reagent), N-succinimidyl S-acetyl thioacetate which is followed by deprotection with hydroxylamine. Additionally, S-acetylmercaptopsuccinic anhydride (SAMSA), N-succinimidyl-3(2-pyridyl dithio) propionate
35

(SPDP) and derivatives thereof being sulfonated or having an extended spacer arm, or 4-succinimidoyloxy carbonyl α -methyl α (2-pyridyl dithio)-toluene (SMPT), can be used, each of which is deprotected by treatment with a reducing agent. A preferred sulphydryl activating reagent is 2-iminothiolane.

5 Binding reagents that can be used within the present invention include, but are not limited to, streptavidin/avidin, lectins, enzymes, glycoproteins, peptides, hormones, receptors, antigens, drugs, antibodies, RNA, DNA, oligonucleotides, or any other selectively bindable molecules 10 or compositions by chemical crosslinking means. Preferred binding reagents are antibodies and antigens. Binding reagents are chemically modified to make them sulphydryl-reactive. Sulphydryl-reactive heterobifunctional crosslinking reagents and sulphydryl-reactive homobifunctional crosslinking reagents are used for this purpose.

15 Examples of heterobifunctional reagents that introduce maleimide groups include, but are not limited to, succinimidyl 4-(N-maleimidomethyl) cyclohexane (SMCC), sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC), *m*-maleimido benzoyl-N-hydroxy succinimide ester (MBS) and the sulfonated derivative of MBS 20 (sulfo-MBS), succinimidyl 4-(*p*-maleimidophenyl) butyrate (SMPB) and the sulfonated derivative thereof, (sulfo-SMPB), and succinimidyl-6-(N-maleimido- -hexanoate) (MHS).

25 Examples of heterobifunctional reagents useful to introduce α -halocarbonyl groups include, but are not limited to, succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), and the sulfonated derivative thereof (sulfo-SIAB), and N-hydroxy succinimidyl bromoacetate (HSBA).

30 Examples of homobifunctional reagents useful for sulphydryl reactive crosslinking include, but are not limited to, bis (maleimido)-methyl ether (BMME) and bismaleimido hexane (BMH). A preferred reagent is sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC).

35 In a preferred embodiment of the invention, recombinant aequorin is chemically modified with 2-iminothiolane, rendering it sulphydryl-activated. Separately, a binding reagent such as an antibody capable of binding specifically to the analyte to be assayed is chemically modified with the heterobifunctional crosslinker sulfo-SMCC to render the binding reagent

sulphydryl-reactive. Subsequently, the sulphydryl-activated photoprotein and sulphydryl-reactive binding reagent are reacted under conditions sufficient to promote coupling to form a photoprotein-binding reagent conjugate.

5 The resulting conjugates optionally may be separated from the unreacted components of the reaction mixture by means commonly known to one skilled in the art. For example, the photoprotein-binding reagent conjugates generally will be appreciably larger in size than either the photoprotein or binding reagent alone. They easily can be purified, 10 therefore, by separation over a chromatography column that separates components of a complex mixture based on their size. Additionally, conjugates may be separated by other means such as ion-exchange chromatography. Such separation techniques are well known in the art.

15 The photoprotein-binding reagent conjugates of the present invention may be used in a variety of binding assays to determine the presence or amount analyte capable of being selectively bound to by a binding reagent. Examples of binding assays include sequential capture assays (also known as sandwich assays), simultaneous capture assays, 20 competitive assays, and nucleic acid hybridization such as oligonucleotide binding to DNA or RNA. While such assays generally are termed binding assays, they may be termed immunoassays when antibodies are involved.

25 In one embodiment, assays of the present invention are conducted by contacting a photoprotein-binding reagent conjugate capable of binding an analyte, with a sample suspected of containing the analyte to be determined, allowing the conjugate and analyte to bind together to form a photoprotein-binding reagent-analyte complex, separating the complex from the rest of the sample, and determining the presence or amount of analyte by initiating a luminescent reaction with the photoprotein and measuring the light produced.

30 Alternatively, the presence and amount of an analyte can be determined in a competitive binding assay wherein a sample with an unknown amount of analyte is combined with a known amount of an analyte-photoprotein conjugate, and the mixture allowed to bind to a limited amount of the binding partner for the analyte. The analyte-photoprotein conjugate competes with the analyte in the sample for binding to the analyte binding partner. The amount of conjugate that binds to the analyte binding 35

partner is determined by initiating the luminescent reaction and measuring the light emitted. The amount of analyte in the sample is then determined as a function of the amount of competitive binding that occurred.

Many diagnostic assays utilize immunoassay techniques, and photoprotein-binding reagent conjugates of the present invention can be prepared where the binding reagent is an antibody. The antibody selected 5 may be either monoclonal or polyclonal, and is capable of binding specifically to the analyte to be assayed. A sample to be assayed, for example, a body fluid, is contacted with a capture reagent, often an antibody, that is immobilized to a solid substrate such as a microtiter well. Analyte in the sample is selectively captured by the capture reagent and non-binding sample is washed away. Subsequently, photoprotein-antibody 10 conjugate is contacted with the captured analyte so as to specifically bind to it. Excess photoprotein-antibody conjugate is washed away. Alternatively, the capture assay is conducted by mixing all binding components 15 simultaneously.

The amount of conjugate bound is determined by measuring amount 20 of light emitted by the conjugate. In the case of aequorin, light production is triggered by the addition of a divalent cation such as calcium. The amount of conjugate determined is indicative of the amount of analyte present in the sample.

The above-described conjugate is an example of the conjugates 25 encompassed by the present invention. Various other binding reagent reagents can be substituted for the antibody. For example, an antigen-conjugate is within the scope of the present invention and is useful in competitive assays for determining the amount of the unconjugated antigen 30 in a sample. Similarly, the conjugates of the present invention encompass lectins as the binding reagent, and are useful for determining the amount and type of carbohydrate or glycoprotein present in a sample. Additionally, the conjugates of the present invention encompass receptors and ligands as the 35 binding reagent, and are useful for determining the presence and amount of a ligand or receptor, respectively, in a sample. Still further, the conjugates of the present invention encompass streptavidin/avidin as the binding reagent, and are useful for determining the presence and amount of any biotinylated analyte. In a similar fashion, the conjugates of the present invention encompass DNA, RNA, and oligonucleotides as the binding

reagent, and are useful for detecting the presence and amount of nucleic acid molecules in a sample by nucleotide hybridization methods known in the art.

Other assays and methods of use of the conjugates of the present invention will be apparent to one skilled in the art and are intended to be encompassed within the appended claims.

The present invention will be better understood in light of the following specific examples.

10 **Example 1**

Prior Attempts That Failed To Make Useful Conjugates

Prior to the invention a number of different approaches for the synthesis of aequorin conjugates were tried. One such approach was the introduction of maleimido groups into aequorin using the heterobifunctional reagent sulfo-SMCC, which contains a primary amine reactive N-hydroxysulfosuccinimide and a thiol reactive maleimide. This strategy involves reaction of aequorin amino groups with the NHS ester portion of sulfo-SMCC, generating a maleimido-activated derivative which subsequently can be covalently coupled with available sulfhydryl groups on a coupling partner. Modification of amino groups *per se* is not deleterious to aequorin, as illustrated by the successful creation of a biotinylated aequorin derivative (Stults et al., 1992).

However, introduction of maleimido groups that can react with accessible sulfhydryl groups on the same or adjacent molecules would likely result in loss of photoprotein activity. At least one of the three cysteine groups in aequorin has been shown to be essential for calcium dependent light production (Shimomura et al., 1978; Kemple et al., 1984). Site-directed mutagenesis studies have confirmed that all three cysteine groups play a role in aequorin activity (Tsuji et al., 1986; Kurose et al., 1989). One skilled in the art would expect, therefore, that chemical modification of critical sulfhydryl groups would significantly compromise the activity of aequorin.

Because at least one of the three cysteine residues in aequorin is involved in light production, and because the maleimido portion of sulfo-SMCC would react with the free sulfhydryl groups on aequorin during the activation step, one skilled in the art would expect a substantial reduction in

aequorin light production activity. It was expected, therefore, that treatment of aequorin with sulfo-SMCC resulted in rapid inactivation of the photoprotein.

Aequorin was treated with up to a three fold molar excess of sulfo-SMCC for 30 minutes at room temperature. Light activity was monitored using a luminometer upon injection of calcium. The drastic and detrimental inactivation of aequorin by treatment with sulfo-SMCC is illustrated in Figure 1. These results confirm the Erikaku et al. observation of rapid and substantial inactivation of aequorin by sulfo-SMCC activation.

In light of these considerations and the above-discussed results, it was not unexpected that the alternative coupling strategy using sulfo-SMCC, wherein the maleimido-activated coupling partner is reacted directly with the free sulphydryls of aequorin, also yielded an unstable conjugate having little photoprotein activity. Table 1 illustrates the dramatic differences between conjugates made with unmodified aequorin and conjugates made using sulphydryl-activated aequorin. Known amounts of thyroid stimulating hormone (TSH) were added to polystyrene tubes coated with a mouse monoclonal antibody against human TSH. Bound hormone was detected with an anti-human TSH mouse monoclonal antibody-aequorin conjugate prepared with aequorin which had been activated with 2-iminothiolane versus aequorin which was not sulphydryl-activated. After washing, light activity was monitored using a luminometer upon injection of calcium.

Table 1
Use Of Antibody-Aequorin Conjugate For The Detection Of Thyroid Stimulating Hormone In A Simultaneous Capture Immunoassay.

| Conjugate | Specific Activity (RLU/OD) | IU/TSH | | |
|---------------------|-------------------------------|-----------|-------------|---------------|
| | | 0 | 0.5 | 50 |
| RLU Bound | | | | |
| Activated Aequorin | 4.2×10^{11} | 232 (6.7) | 7163 (16.5) | 333368 (2.1) |
| Unmodified Aequorin | 3.1×10^{10} | 266 (4.5) | 1603 (7.2) | 106068 (12.7) |

The unit μ IU is micro International units of TSH, RLU means relative light units, per RIU/OD means relative light units per optical density at 280 nm, and the number in parentheses is the %CV (coefficient of variation).

5 It is important to note that 6.5 fold more conjugate was required in the assays using conjugate prepared with unmodified AEQ. This data demonstrates that the performance of the conjugates synthesized with unmodified aequorin is very inferior to conjugates synthesized using aequorin modified to contain additional sulphydryl groups. The specific activity of conjugates prepared without sulphydryl addition is less than 10% compared to conjugates prepared with 2-iminothiolane. While, the conjugate made with non-modified aequorin binds to TSH in an immunoassay, its performance is limited both in terms of immunoreactive counts, amount of IgG that has to be added, and the overall performance of 15 the assay.

10 The discovery of modified and crosslinked aequorin-antibody conjugates that retained substantial luminescence was, therefore, a surprising and unexpected result. The highly photoactive aequorin-antibody conjugates of the present invention were synthesized as described below. 15 The following non-limiting example is provided so that one skilled in the art can better understand the invention as defined by the appended claims.

Example 2

Synthesis of Conjugates of Sulphydryl-Activated Aequorin

20 **Materials.** 2-iminothiolane and sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) were obtained from Pierce Chemical Co. DEAE-Sepharose, Sephadex, Sephadex, and all other chemicals were obtained from either Sigma Chemical Co. or Fisher Scientific. Light measurements were made using either a tube (Berthold Clinilumat LB9502) or microtiter plate (Dynatech ML1000 Microplate) 25 luminometer. Opaque microtiter wells available from Dynatech (Microlite 2 wells) were used in the Dynatech instrument.

30 **Activation of Aequorin with 2-iminothiolane.** Aequorin, which is routinely stored in 10 mM Tris, 0.15 M NaCl, pH 8.0 containing 2 mM EDTA (TBS/E), is exchanged into 0.1 M sodium bicarbonate, 0.2 M 35

5 NaCl, 2 mM EDTA, pH 8.5 prior to activation with 2-iminothiolane by
passing over a desalting column equilibrated in the bicarbonate buffer. One
ml fractions are collected and the O.D.280 is monitored. The void fractions
containing aequorin are pooled together and the amount of protein in the
pooled fractions is estimated by O.D.280. Aequorin, which is now in the
bicarbonate buffer, is then reacted with a 2-200 fold molar excess of 2-
iminothiolane, by adding the appropriate volume of freshly prepared stock
solution of 2-iminothiolane in bicarbonate buffer to the pooled aequorin.
The solution is mixed gently and left at room temperature for 30 min. The
reaction is stopped by adding excess amino reagent (50-100 μ l of 1.5 M
10 Tris, pH 7-9). The reaction mixture is passed over a desalting column
equilibrated in degassed 0.1 M Tris, 2 mM EDTA, pH 7.0 (TE) to remove
excess 2-iminothiolane. One ml fractions are collected and the O.D.280 is
monitored. The void fractions containing the 2-iminothiolane activated-
15 aequorin are combined and the amount of protein in the pooled fractions is
estimated by O.D.280. The 2-iminothiolane activated-aequorin is stored
on ice until it is combined with the maleimido-activated protein. Figure 2
demonstrates that sulphydryl activation of aequorin with 2-iminothiolane,
even at a 100 molar excess, does not adversely affect aequorin light activity.
20 Aequorin was treated with the amounts of 2-iminothiolane (Traut's reagent)
indicated in Figure 2 for 30 minutes at room temperature. Light activity was
measured using a luminometer upon injection of calcium.

Activation of antibody. Binding reagents such as antibodies are routinely activated with a 5-50 fold molar excess of sulfo-SMCC. The antibody of interest, if lyophilized, is directly reconstituted in, or if in solution, exchanged by gel filtration, into 0.1 M sodium bicarbonate, 0.2 M NaCl, 0.5 mM EDTA, pH 7.5. The amount of antibody to be activated is determined by protein determination or by O.D.280 using the appropriate molar extinction coefficient. The appropriate volume of freshly prepared stock solution of sulfo-SMCC in bicarbonate is added to the antibody, the solution is mixed gently and allowed to react at room temperature for 30 min. The reaction is stopped by addition of Tris as described above and the reaction mixture is then passed over a desalting column equilibrated in degassed 0.1 M Tris, 2 mM EDTA, pH 7.0 to remove excess sulfo-SMCC. The void fractions containing maleimido-activated antibody are combined and the amount of protein in the pooled fractions is determined.

from the O.D.280. The maleimido activated-antibody is stored on ice until it is combined with the 2-iminothiolane activated aequorin.

Conjugation and Conjugate Activity Assay. The 2-iminothiolane activated aequorin and sulfo-SMCC activated antibody are combined, usually at a molar ratio between 3 and 30, mixed gently, and allowed to react, without agitation, overnight at 4°C. To test for the presence of active antibody conjugate in the reaction mixture, aliquots are incubated in opaque microtiter wells or polystyrene tubes which have been coated with appropriate serum or purified anti-conjugate antibody or antigen which the aequorin-antibody conjugate will recognize or which will capture the conjugate or, in the case of streptavidin/avidin with a biotinylated target. For example, in the case of a mouse monoclonal antibody the microtiter wells can be coated with a goat anti-mouse immunoglobulin which will capture the conjugate. Typically, the wells or tubes are coated with 100 µl of 5-50 µg/ml of antibody overnight at 4°C. The coated wells or tubes are then blocked with an appropriate blocking reagent such as 1% gelatin/5 mg/ml BSA. A parallel set of blank wells or tubes are prepared by incubating untreated opaque microtiter wells with the same blocking reagent.

Several 10-fold dilutions of the conjugate reaction mixture are prepared in TBS/E containing 0.1 mg/ml BSA (TBS/E/BSA). Aliquots (100 µl/well) are placed into the blocked blank wells or tubes and blocked coated wells or tubes and allowed to incubate for 10 or more min at room temperature. The wells are washed 4-5 times with TBS/E to remove unbound conjugate. TBS/E/BSA (50 µl) is added to the wells or tubes to prevent drying. The microtiter wells or tubes are placed in an appropriate luminometer to monitor the presence of bound conjugate upon injection of calcium (10 - 100 mM Tris, 10 - 100 mM calcium acetate, pH 7.5). If a significant light signal is observed in wells or tubes coated with antibody/antigen/biotinylated target compared to blank wells or tubes, the conjugate is purified as follows.

Purification of Conjugate. Conjugates of aequorin with antibodies can be purified using a combination of gel filtration and ion-exchange chromatography. The order in which the purification steps are carried out does not matter with respect to the purified conjugate. Gel filtration is carried out using an appropriate resin (e.g., Sephadex G-100 or

G-150, Sephacryl S-100 or S-200) which serves to separate free aequorin (MW 22,000) from the aequorin-antibody conjugate and any unreacted antibody present in the reaction mixture. An appropriate ion exchange media is used to remove unreacted antibody from the aequorin-antibody conjugate obtained after gel filtration. DEAE-Sepharose is routinely used which binds aequorin due to its acidic isoelectric point (pH-4.5). Aequorin-antibody conjugates are bound by the resin and require a salt gradient for elution. Binding reagents such as antibodies and streptavidin/avidin have more basic isoelectric points than aequorin, and will therefore either run through a DEAE column or will be bound with lesser affinity and will elute at a lower salt concentration.

The conjugate reaction mixture is applied to the gel filtration column equilibrated in TE, pH 7.0. For example, a conjugate with antibody is applied to a Sephacryl S-200 column (1.2 x 85 cm) at 4°C and the column is eluted with TE. Fractions (0.5-1.0 ml) are collected and monitored for the presence of light activity and protein by absorbance at 280 nm. The peak which elutes near the void volume of the column contains aequorin-antibody conjugate as well as unreacted binding reagent. The included peak of light activity corresponds to free aequorin. Figure 4 illustrates a Sephacryl S-200 column chromatography purification profile of antibody-aequorin conjugate. One ml fractions were collected, protein concentration was measured spectrophotometrically at 280 nm, and light activity was measured using a luminometer upon injection of calcium.

The fractions which contain the aequorin-antibody conjugate are pooled and applied to a 5 ml DEAE-Sepharose column (1 x 12 cm) equilibrated with TE, pH 7.0. After washing the DEAE column with about 15-20 ml of TE, pH 7, proteins bound to the column are eluted using a linear salt gradient (50 ml total volume) with 25 ml of TE, pH 7 and 25 ml TE, pH 7 containing 0.6 M NaCl. One ml fractions are collected and monitored for absorbance at 280 nm and light activity. Free antibody will be present in the first protein peak. The aequorin-antibody conjugate will be eluted later as a peak containing protein and light activity.

Figure 5 illustrates the elution profile of purification of antibody-aequorin conjugate by DEAE-Sepharose ion exchange chromatography. Light activity was measured using a luminometer upon injection of calcium. The fractions containing the purified aequorin-antibody are pooled and

5 retested for the presence of active conjugate as described above. An aliquot of the purified conjugate is subjected to SDS-PAGE to further confirm the presence of conjugate. The specific activity (light activity per mg) of the conjugate is determined and the conjugate is stored under appropriate conditions.

Example 3:

Preparation of An Anti-Human Antibody-Aequorin Conjugate.

10 An anti-human TSH monoclonal antibody-aequorin reaction mixture, containing a 12 fold molar excess of sulphydryl-activated aequorin over sulfo-SMCC maleimido-activated mouse anti-TSH antibody retained approximately 84% of its original light activity after overnight coupling at 4°C.

15 The reaction mixture was shown to contain the desired conjugate based on the binding of 3-10 fold more light activity in microtiter wells that had been coated with goat anti-mouse antibody compared to wells that had been treated with blocking reagent only. Dilutions of conjugate were placed in opaque microtiter wells that were coated with goat anti-mouse antibody and treated with blocking reagent for 30 minutes at room temperature.

20 Control microtiter wells were treated with blocking reagent only. After washing away unbound conjugates, bound light activity was determined using a microplate luminometer upon injection of calcium. The specific binding results are shown in Figure 3.

25 The reaction mixture was placed over a Sephadryl S-200 column followed by DEAE-Sepharose ion exchange chromatography. The chromatographic profile is illustrated in Figure 4. As shown in Figure 4, approximately 6% of the light activity and 36% of the protein applied to the S-200 column was eluted near the void volume while the remaining light activity eluted in a position corresponding to free aequorin. The DEAE step served to remove traces of unconjugated antibody as shown in Figure 5.

30 Coupling was confirmed by reducing SDS-PAGE analysis of the purified conjugate in which several bands of molecular weight greater than the antibody heavy chain (50 kD) were observed, data not shown. In addition, the same conjugate was stable in solution and to lyophilization with respect to both photoprotein and immunological activity. The conjugate retained approximately 70% of its light activity after storage in an

appropriate buffer for one month at 4°C and greater than 85% of its light activity when stored as a lyophilized powder at room temperature.

Example 4

Thyroid Stimulating Hormone Binding Assay

5 A thyroid stimulating hormone (TSH) assay was performed. Briefly, known amounts of thyroid stimulating hormone (TSH) were added to polystyrene tubes coated with a mouse monoclonal antibody against human TSH. Bound hormone was detected with an anti-human TSH mouse monoclonal antibody-aequorin conjugate. After washing, light activity was monitored using a Berthold Clinilumat luminometer. As shown in Table 2, the anti-TSH antibody-aequorin conjugate was functional as demonstrated by the ability to detect low levels of TSH in a capture immunoassay.

Table 2

Use Of Antibody-Aequorin Conjugate For The Detection Of Thyroid Stimulating Hormone In A Simultaneous Capture Immunoassay.

| μIU^2 /ml | Relative Light Units (RLU) | %CV |
|-------------------------|----------------------------------|-----|
| 0 | 137 | 5.1 |
| 0.1 | 2458 | 1.2 |
| 50.0 | 658782 | 2.7 |

μIU = micro International units of TSH.

Example 5

Competitive Immunoassay

A competitive immunoassay for thyroxine using thyroxine-(T4) aequorin conjugate was performed. Known amounts of thyroxine (T4) were added to polystyrene tubes coated with a donkey antibody against mouse IgG containing buffer with both a mouse antibody against thyroxine

(T4) and a thyroxine-(T4) aequorin conjugate. After washing, bound conjugate was determined upon detection of calcium using a luminometer. The resulting competitive binding curve is shown in Figure 6.

5 **Example 6**

Preparation of nucleic acid photoprotein conjugates

In general DNA/RNA probes can be derivatized in manners similar to proteins. Complementary probes to specific sequences of RNA and DNA can be made and derivatized either directly through the amino terminal or side chain amino, carboxyl, or other functionality, or reacted with derivatizing agents to provide more active "labelable" groups. In addition DNA can be cross linked with materials such as psoralens that can provide free amine groups for attachment. Examples illustrating these methods follow.

15 Probes can be reacted with activators such as 1, 1'-carbonyldiimidazole and a bis-amine to provide an aliphatic primary amine attachment for the probe that does not interfere with DNA double helix formation. The primary amine can then be reacted, as the oligonucleotide, with a NHS ester, such as Sulfo-SMCC, to add the maleimido function. Examples of this are shown in the paper by S. Beck, T. O'Keeffe, J. J. Coull, and H. Küster. (Nuc. Acid. Res., 1989, 17, 5115-5123), which is incorporated by reference. While the modified oligo is reacted with NHS-biotin, it is substantially likely that SMCC could be used.

20 Alkyl amine linkers can be added either during synthesis of the probe or post synthesis. An example of this modification is the use of allyl amine. This will react with, for example, the C-5 position of pyrimidines to yield the allyl amine group which can be modified using standard NHS or other chemistry to attach a SMCC or equivalent group. See L. Gardner in BioTechniques, 1983, 38-41, and their use in PNAS, 1983, 80, 4045-4049, both of which are incorporated by reference.

25 Acridinium esters have been attached to amine modified DNA and RNA molecules using NHS chemistry to yield DNA and RNA probes. See L. J. Arnold Jr., in Clinical Chemistry, 1989, 35, 1588-94, which is incorporated by reference.

30 Such probes can be used in nucleic acid hybridization assays, such as oligonucleotides hybridizing to DNA or RNA, that are known in the art.

Having fully described the invention in the above description and examples, other variants and uses will be apparent to one skilled in the art. All such variations are intended to fall within the scope of the appended claims.

I Claim:

Claims

- 5 1. A photoprotein-binding reagent conjugate composition comprising a sulphydryl-activated photoprotein capable of participating in a luminescent reaction to produce light, coupled to a sulphydryl-reactive binding reagent capable of binding specifically to an analyte.
- 10 2. The photoprotein-binding reagent of Claim 1, wherein said sulphydryl-activated photoprotein is modified to contain at least one sulphydryl group more than is present in the unmodified photoprotein, and wherein said sulphydryl-reactive binding reagent is modified so as to crosslink with a sulphydryl group on said photoprotein, the resulting photoprotein-binding reagent conjugate capable of binding specifically to said ligand, and capable of participating in a luminescent reaction to produce light.
- 15 3. The composition of Claim 1 wherein said photoprotein is selected from the group consisting of apoaequorin, aequorin, apo-obelin, obelin, apo-mnemiopsin, mnemiopsin, apo-berovin, berovin, pholasin, luciferases, and bioluminescent proteins isolated from *Pelagia*, or ostracods.
- 20 4. The composition of Claim 1 wherein said binding reagent is selected from the group consisting of streptavidin/avidin, lectins, enzymes, glycoproteins, peptides, hormones, receptors, antigens, drugs, antibodies, RNA, DNA, and oligonucleotides.
- 25 5. The composition of Claim 1 wherein said photoprotein is apoaequorin or aequorin and said binding reagent is an antibody.

6. The composition of Claim 1 wherein said photoprotein is apoaequorin or aequorin and said binding reagent is an antigen.

5 7. The composition of Claim 1 wherein said photoprotein is apoaequorin or aequorin and said binding reagent is a nucleic acid molecule.

10 8. The composition of Claim 1 wherein said photoprotein is sulfhydryl activated with a sulfhydryl activating reagent selected from the group consisting of 2-iminothiolane (Traut's reagent); N-succinimidyl S-acetyl thioacetate which is followed by deprotection with hydroxylamine; S-acetylmercaptopsuccinic anhydride (SAMSA) followed by deprotection by treatment with a reducing agent; N-succinimidyl-3-(2-pyridylidithio) propionate (SPDP) and derivatives thereof, followed by deprotection by treatment with a reducing agent; and 4-succinimidylcarbonyl α -methyl α -(2-pyridylidithio)-toluene (SMPT) followed by deprotection by treatment with a reducing agent.

15

9. The composition of Claim 8 wherein said photoprotein is sulfhydryl activated with 2-iminothiolane.

20 10. The composition of Claim 1 wherein said binding reagent is made sulfhydryl reactive by modifying said binding reagent to contain a maleimido group or α -halocarbonyl group.

25 11. The composition of Claim 1 wherein said binding reagent is made sulfhydryl reactive by treating said binding reagent with a compound selected from the group consisting of succinimidyl 4-(N-maleimidomethyl) cyclohexane, (SMCC) the sulfonated derivative of SMCC *m*-maleimido benzoyl-N-hydroxy succinimide ester (MBS), the sulfonated derivative of MBS (sulfo-MBS), succinimidyl 4-(*p*-maleimidophenyl) butyrate (SMPB), the sulfonated derivative of SMPB (sulfo-SMPB), succinimidyl-6-(N-maleimide-*n*-hexanoate), succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), the sulfonated derivative of SLAB (sulfo-SIAB), N-hydroxy succinimidyl bromoacetate, bis-(maleimido) methyl ether, and bis-maleimido hexane (BMH).

30

35

12. The composition of Claim 11 wherein said binding reagent is made sulphydryl reactive by treating said binding reagent with sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo SMCC).

5

13. A method of making a photoprotein-binding reagent conjugate composition comprising the step of coupling a sulphydryl-activated photoprotein capable of participating in a luminescent reaction to produce light, to a sulphydryl-reactive binding reagent capable of binding specifically to an analyte.

10

14. The method of Claim 13, wherein said sulphydryl-activated photoprotein is modified to contain at least one sulphydryl group more than is present in the unmodified photoprotein, and wherein said sulphydryl-reactive binding reagent is modified so as to a crosslink to a sulphydryl group on said photoprotein, the resulting photoprotein-binding reagent conjugate capable of binding specifically to said ligand, and capable of participating in a luminescent reaction to produce light.

15

15. The method of Claim 13, wherein said photoprotein is selected from the group consisting of apoaequorin, aequorin, apo-obelin, obelin, apo-mnemiopsin, mnemiopsin, apo-berovin, berovin, pholasin, luciferases, and bioluminescent proteins isolated from *Pelagia*, or ostracods.

20

16. The method of Claim 13 wherein said binding reagent is selected from the group consisting of streptavidin/avidin, lectins, enzymes, glycoproteins, peptides, hormones, receptors, antigens, drugs, antibodies and antigen binding fragments thereof, RNA, DNA, and oligonucleotides.

25

17. The method of Claim 13 wherein said photoprotein is apoaequorin or aequorin and said binding reagent is an antibody.

18. The method of Claim 13 wherein said photoprotein is apoaequorin or aequorin and said binding reagent is an antigen.

19. The method of Claim 13 wherein said photoprotein is apoaequorin or aequorin and said binding reagent is a nucleic acid molecule.

5 20. The method of Claim 13 wherein said photoprotein is sulphhydryl activated with a sulphhydryl activating reagent selected from the group consisting of 2-iminothiolane (Traut's reagent); N-succinimidyl S-acetyl thioacetate which is followed by deprotection with hydroxylamine; S-acetylmercaptosuccinic anhydride (SAMSA) followed by deprotection by treatment with a reducing agent; N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and derivatives thereof, followed by deprotection by treatment with a reducing agent; and 4-succinimidylcarbonyl α -methyl α (2-pyridyldithio)-toluene (SMPT) followed by deprotection by treatment with a reducing agent.

15 21. The method of Claim 20 wherein said photoprotein is sulphhydryl activated with 2-iminothiolane.

20 22. The method of Claim 13 wherein said binding reagent is made sulphhydryl reactive by modifying said binding reagent to contain a maleimido group or α -halocarbonyl group.

25 23. The method of Claim 13 wherein said binding reagent is made sulphhydryl reactive by treating said binding reagent with a compound selected from the group consisting of succinimidyl 4-(N-maleimidomethyl) cyclohexane, (SMCC) the sulfonated derivative of SMCC *m*-maleimido benzoyl-N-hydroxy succinimide ester (MBS), the sulfonated derivative of MBS (sulfo-MBS), succinimidyl 4-(*p*-maleimidophenyl) butyrate (SMPB), the sulfonated derivative of SMPB (sulfo-SMPB), succinimidyl-6-(N-maleimide-*n*-hexanoate), succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), the sulfonated derivative of SIAB (sulfo-SIAB), N-hydroxy succinimidyl bromoacetate, bis-(maleimido) methyl ether, and bis-maleimido hexane (BMH).

24. The method of Claim 23 wherein said binding reagent is made sulphydryl reactive by treating said binding reagent with sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC).

5

25. An assay method comprising determining the presence or amount of an analyte in a sample by using the photoprotein-binding reagent conjugate of Claim 1 in a binding assay.

10

26. The assay method of Claim 25 wherein the binding assay is selected from the group comprising simultaneous capture binding assays, sequential capture binding assays, competitive binding assays, and nucleic acid hybridization.

15

27. The assay method of Claim 25 wherein the photoprotein-binding reagent conjugate capable of binding specifically to the analyte to be determined is contacted with the sample suspected of containing the analyte to be determined, under condition sufficient for the photoprotein-binding reagent conjugate and analyte to bind together to form a photoprotein-binding reagent-analyte complex, simultaneously or subsequently separating the complex from the rest of the sample, and determining the presence or amount of analyte by initiating a luminescent reaction with the photoprotein and measuring the light produced.

25

28. The assay method of Claim 25 wherein the photoprotein-binding reagent conjugate capable of selectively binding to the binding partner of the analyte to be determined is contacted with the sample suspected of containing the analyte to be determined, under condition sufficient for the photoprotein-binding reagent conjugate and binding partner of the analyte to bind together to form a photoprotein-binding reagent-analyte binding partner complex, subsequently separating the complex from the rest of the sample, and determining the presence or amount of analyte by initiating a luminescent reaction with the photoprotein and measuring the light produced.

30

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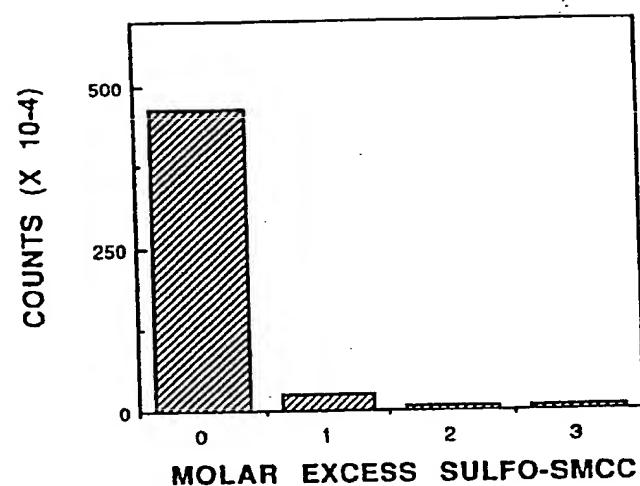


FIGURE 1

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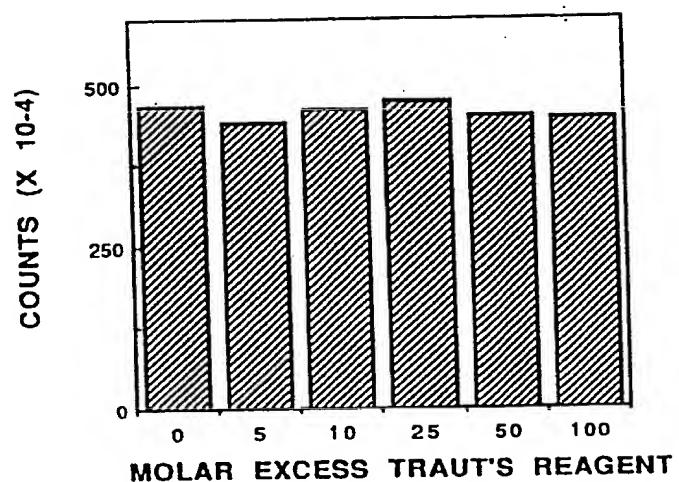


FIGURE 2

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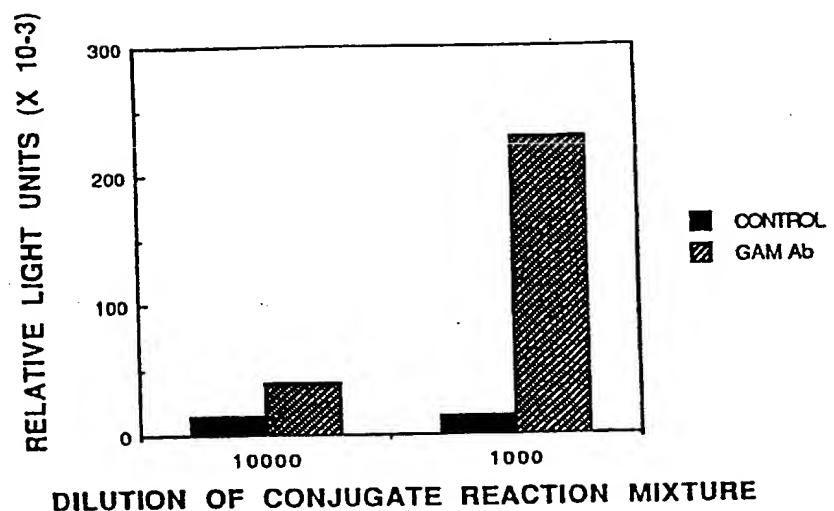


FIGURE 3

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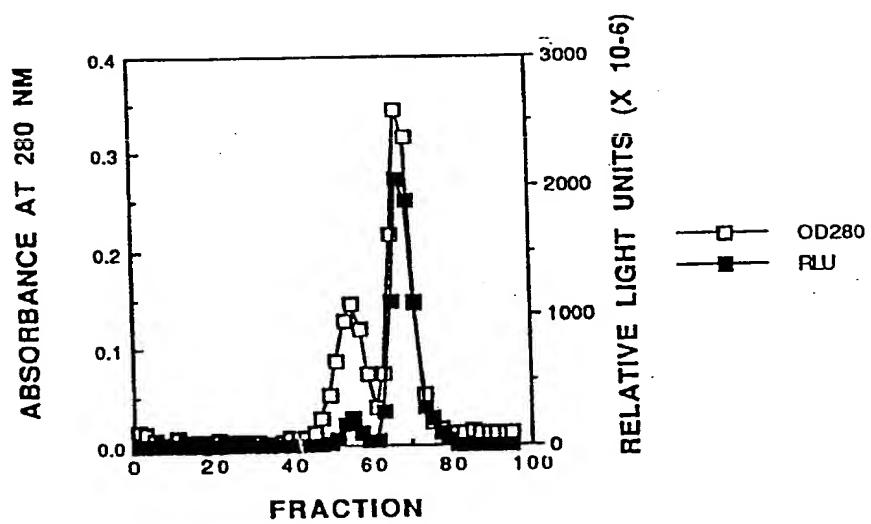


FIGURE 4

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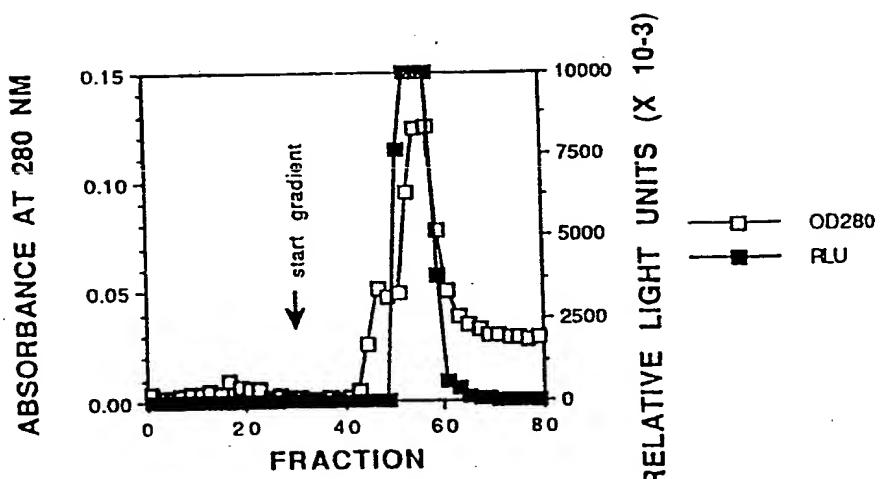


FIGURE 5

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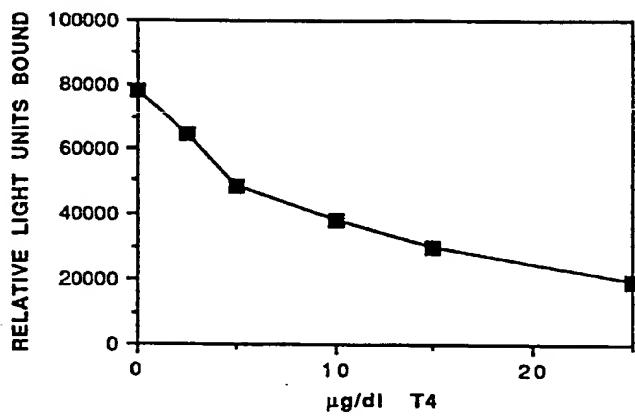


FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/01387

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12Q 1/00, 1/66

US CL : 435/4, 8, 7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 8, 7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS
aequorin, chemiluminescence, bioluminescence, antibody, apoaequorin, photoprotein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| Y | US, A, 5,094,939 (OKADA ET AL.) 10 March 1992. | 1-28 |
| Y | US, A, 5,098,828 (GEIGER ET AL.) 24 March 1992. | 1-28 |
| Y | US, A 4,665,022 (SCHAEFFER ET AL.) 12 May 1987. | 1-28 |
| Y | BIOCHEMISTRY, Volume 17, No. 8, issued 1978, T.P. King et al., "Preparation of protein conjugates via intermolecular disulfide bond formation", pages 1499-1506, see entire document. | 1-28 |

 Further documents are listed in the continuation of Box C. See patent family annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/01387

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 174, No. 3, issued 14 February 1991, T. Erikaku et al., "Bioluminescent immunoassay using a monomeric Fab'-photoprotein aequorin conjugate", pages 1331-1336, see entire document. | 1-28 |
| Y | PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 86, issued January 1989, K. Kurose et al. "Bioluminescence of the Ca^{2+} -binding photoprotein aequorin after cysteine modification", pages 80-84, see entire document. | 1-28 |
| Y | NATURE, Volume 338, no. 6216, issued 13 April 1989, I. Bronstein et al., "Chemiluminescence lights up", pages 599-600, see entire document. | 1-28 |
| Y | BIOCONJUGATE CHEMISTRY, Volume 1, Number 1, issued 1990, G.E. Means et al., "Chemical Modifications of Proteins: History and Applications", pages 2-12, see entire document. | 1-28 |

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